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Journal of Chromatography A, 922 (2001) 365–369

JOURNAL OF CHROMATOGRAPHY A

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Short communication

Separation of chondroitin sulfate and hyaluronic acid fragments by centrifugal precipitation chromatography

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Received 17 April 2001; accepted 10 May 2001

Abstract

Centrifugal precipitation chromatography (CPC) was applied for the first time to the separation of fragments of chondroitin sulfate (ChS) and hyaluronic acid (HA). The separation was performed using a gradient elution system between ethanol and water since solubility of these biopolymers highly depends on the concentration of ethanol in aqueous solution. ChS and HA were each eluted into several peaks through a flow-through UV detector at 275 nm, despite they have almost no absorbance at this wavelength in an aqueous solution. The separation was also confirmed by redissolving the dried fraction in water and measuring the absorbance at 210 nm. These results suggest that the CPC system can detect small precipitates of these biopolymers by light scattering at 275 nm. The separated fragments of biopolymers are not easily characterized because no suitable analytical method is available for identification of these compounds. However, the overall results demonstrate that CPC may be a useful separation of biopolymers such as glycosaminoglycans which quantitatively produce precipitates in an organic solvent mixture.

Keywords: Centrifugal precipitation chromatography; Chondroitin sulfate; Hyaluronic acid; Glycosaminoglycans; Carbohydrates

1. Introduction

Chondroitin sulfates (ChSs) and hyaluronic acid (HA) are glycosaminoglycans (GAGs) mainly contained in the connective tissue. ChSs are made from a series of disaccharide units composed of glucuronic acid and *N*-acetylgalactosamine with a molecular mass of ca. 50 000, while HA is made by glucuronic acid and *N*-acetylglucosamine with a molecular mass of ca. 1 000 000. ChSs generally form heterogeneous structures due to the differences in binding portion of sulfonate groups of *N*-acetylgalactosamine moiety. Recently, the biological activity of these compounds is gradually revealed, especially in low molecular mass of HA for angiogenesis. The chemical structures of these compounds are shown in Fig. 1.

Various fractionation methods to purify GAG from

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0021-9673/01/$ – see front matter
PII: S0021-9673(01)00959-1

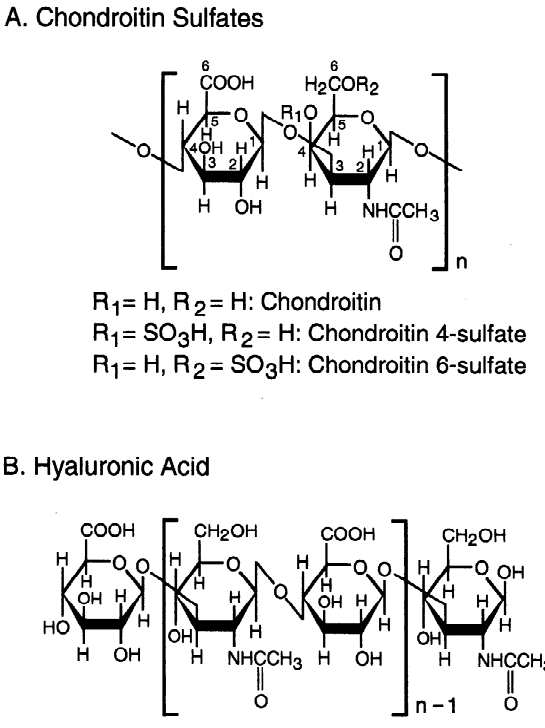

Fig. 1. Chemical structures of chondroitin sulfates and hyaluronic acid.

crude biological materials have been reported in the past [1]. Most of these methods are mainly based on the difference of their physico-chemical characteristics such as solubility in the suitable solvent mixture, charge density and molecular mass. Among these methods, the precipitation method with ethanol can be used to isolate GAGs from biological samples. Meyer et al. [2] reported the fractionation of dermatan sulfate, chondroitin 4-sulfate and chondroitin 6-sulfate from crude mucopolysaccharide samples at various concentrations of ethanol in calcium acetate–acetic acid buffer solution. Jorpes and Gardell [3] also reported the separation between ChS and heparan sulfate as barium salts using the ethanol precipitation method. However, the separation of highly purified fragments of individual GAG has not been reported probably due to a lack of a suitable separation technique.

Recently, Ito developed a new separation method called centrifugal precipitation chromatography (CPC) which depends on the solubility of solutes in solvents [4,5]. It has been demonstrated that this CPC system is useful for separating biopolymers such as proteins [4,5] and polyphenols [6]. The present study describes the separation of ChS and HA fragments using the CPC separation technique with a gradient elution system between ethanol and water.

2. Experimental

2.1. Apparatus

The separation disk assembly for CPC was fabricated at the machine stop of the National Institutes of Health, Bethesda, MD, USA. The separation principle and basic design are described elsewhere [4,5]. The separation column used in the present study was made of a pair of flat disks (high density polyethylene, 13.2 cm diameter and 1.5 cm in thickness) with a spiral-shaped narrow groove (1.5 mm wide and ca. 2 m in length). The spiral groove of the upper disk (0.5 mm deep) is made in a mirror image to that of the lower disk (2 mm deep) so that with a proper alignment these grooves form a single channel. A dialysis membrane sheet (regenerated cellulose, molecular mass cut-off 12 000–14 000, Spectrum, Laguna Hills, CA, USA) is sandwiched between these two disks to form two channels. The capacity of upper and lower channels is 2 ml and 7.4 ml, respectively. The disk assembly is tightly bolted with the leakage-free seal and mounted on the sealless continuous-flow centrifuge (Pharma-Tech, Baltimore, MD, USA) which allows elution through multiple channels without the use of rotary seals [7,8].

2.2. Reagents

A set of commercially purified ChS and HA samples was obtained from Seikagaku (Tokyo, Japan). All others reagents were of reagent grade.

2.3. Procedure for fractionation

The entire elution system of the present method is schematically illustrated in Fig. 2. In each experiment, both the ethanol channel (lower channel) and the water channel (upper channel) were completely

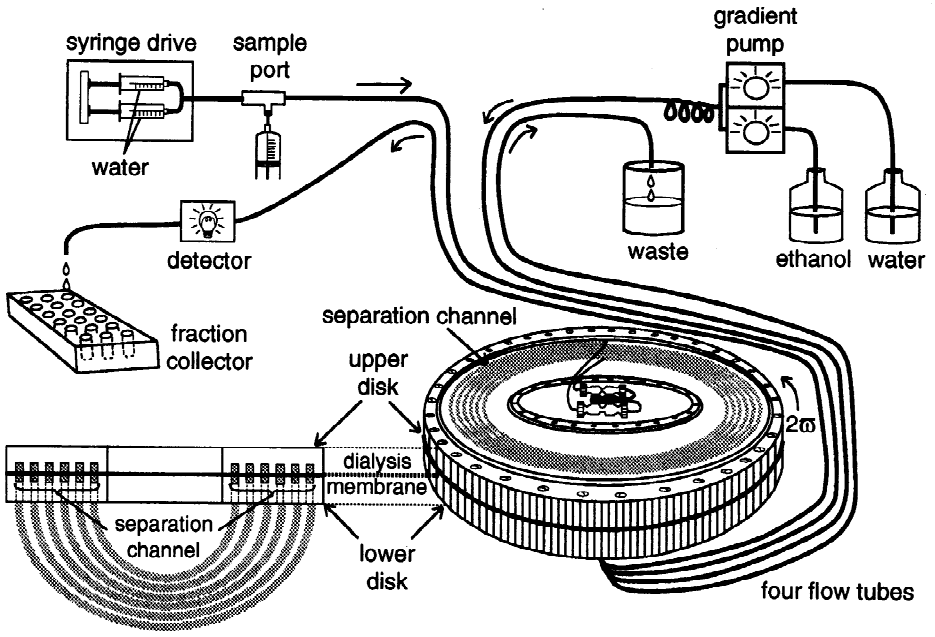

Fig. 2. Schematic drawing of the centrifugal precipitation chromatography system used in the present studies.

filled with absolute ethanol. After the sample solution was introduced via a sample loop the column was rotated at 2000 rpm. Then, the lower channel was eluted with the increase of water concentration in ethanol using a gradient pump (Shimadzu SCL-10A and LC-10AD, Shimadzu, Columbia, MD, USA) at a total flow-rate of 1 ml/min. On the other hand, the upper channel was eluted with water using a syringe drive (Model 980532, Harvard Apparatus, South Natick, MA, USA) at a flow-rate of 0.06 ml/min. The gradient elution was programmed as follows: absolute (100%) ethanol for 0.5 h, then a linear gradient from 100% to 50% for 6 h. The effluent from the upper channel was continuously monitored through an UV monitor (Uvicord S, LKB Instruments, Stockholm, Sweden) at 275 nm and collected into test tubes at 20-min intervals using a fraction collector (Ultrorac, LKB Instruments). The elution curve was traced with a strip chart recorder (Pharmacia LKB RC102, Stockholm, Sweden).

2.4. Measurement of ^{1}H-NMR spectra

The fractionated sample solution was dried and dissolved in D_2O (99.96%) and then transferred into a NMR tube (25 cm×5.0 mm O.D., pp-528; Wilmad Glass, Buena, NJ, USA). One-dimensional (1D) ^{1}H-NMR experiments were performed on a JEOL GSX500A spectrometer equipped with a 5 mm field gradient tunable probe with standard JEOL software at 303 K for nuclear overhauser effect (NOE) spectra of 333 K for other experiments on 500 μl samples. The proton deutron oxide, water (HOD) signal was suppressed by presaturation during 3 s for 1D experiments.

3. Results and discussion

GAGs (ChS and HA) are conventionally purified using ethanol precipitation method from biological materials. However, this method requires further fractionation of each GAG from the precipitate including total GAGs. The purified GAG commonly contains various sugar chains composed of different molecular masses at relatively wide ranges. Therefore, a new separation method is required to separate these sugar chains individually based on their physiological characteristics.

The CPC system used in the present study is a

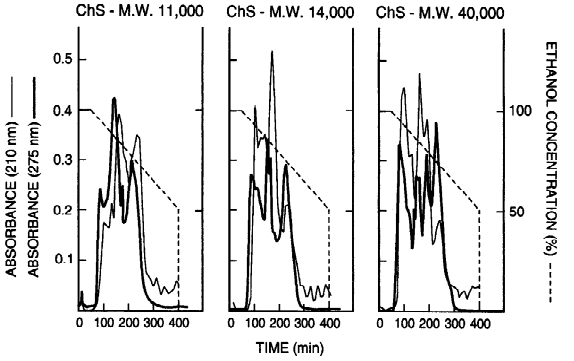

Fig. 3. Chromatograms of ChS fragments obtained by centrifugal precipitation chromatography. Experimental conditions: Apparatus, sealless continuous-flow centrifuge; separation column, a pair of high-density polyethylene disks (13.2 cm diameter and 1.5 cm thick); separation channel, 1.5 mm wide and 0.5 mm deep for upper disk, 1.5 mm wide and 2 mm deep for lower disk partitioned with a dialysis membrane (M_r ca. 6000–8000, 100-μm-thick); upper channel, water at a flow-rate of 0.06 ml/min; lower channel, ethanol gradient, 100% for 0.5 h, a linear gradient 100–50% for 6 h at a flow-rate of 1.0 ml/min; sample, 5 mg dissolved with 1 ml of distilled water; revolution, 2000 rpm; detection, 275 nm.

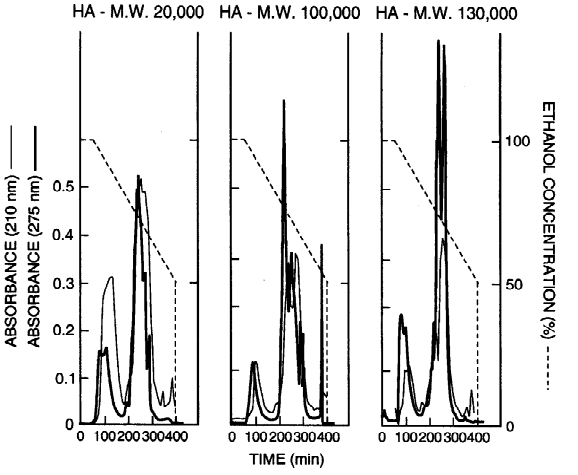

Fig. 4. Chromatograms of HA fragments obtained by centrifugal precipitation chromatography. Experimental conditions are the same as those in Fig. 3.

novel chromatographic technique to separate high molecular compounds according to their solubility in the suitable solvent mixture. Fig. 3 illustrates the chromatograms of various ChS using the CPC system. ChSs were eluted into several peaks along the linear gradient between ethanol and water.

On the other hand, all HA samples are eluted into two distinct fractions at the almost same elution time regardless of their different molecular masses (20 000–130 000) under the same experimental conditions as those of ChS shown in Fig. 4. The first fraction was eluted at 100% ethanol concentration in the lower channel of the separation disk, and the second peak fraction was eluted at a relatively hydrophilic solvent condition. In order to reveal the difference between two fractions, 1D ^{1}H-NMR spectra were measured using a HA sample at the molecular mass of 20 000 as shown in Fig. 5. The remarkable difference between two peak fractions is found in the peak around 3.9 ppm, which corresponds to the protonated carboxyl group in the glucuronic acid moiety of HA molecule. These results suggest that the first fraction is hydrophilic HA fragments since its dissociated carboxyl group

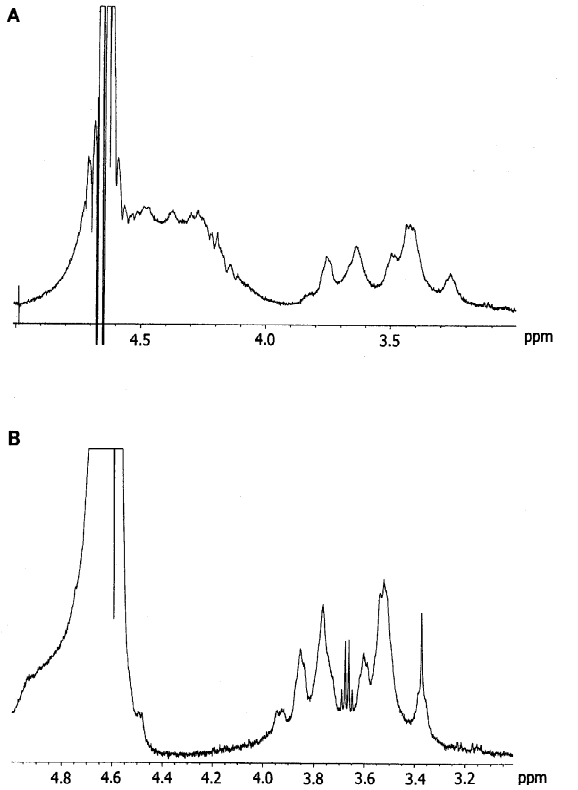

Fig. 5. One-dimensional ^{1}H-NMR spectra of hyaluronic acid (M_r=20 000) fragments obtained by centrifugal precipitation chromatography. (A) First peak fractions; (B) second peak fractions.

gives no peaks around 3.9 ppm (Fig. 5A), and the second fraction is hydrophobic HA fragments (Fig. 5B). Scott has proposed that HA formed hydrophobic regions over the wide range in the molecule [9]. If this is the case, the present CPC system can chromatographically separate HA fragments according to their hydrophobicity. However, the separated fragments are not easily characterized because no suitable analytical methods are available for identification of these compounds.

Both ChS and HA fragments were detected with a flow-through UV detector at 275 nm, despite these compounds having almost no absorbance at the same wavelength in an aqueous solution. The separation was also confirmed by redissolving the dried fraction in water and measuring the absorbance at 210 nm. These results suggest that the CPC system can detect small precipitates of these biopolymers by light scattering at 275 nm.

As described above, the overall results demonstrate that CPC may be useful for separation of biopolymers such as GAGs, which quantitatively produce precipitates in an organic solvent mixture.

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